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(54) Title: VACCINES CONTAINING PAUCILAMELLAR LIPID VESICLES AS IMMUNOLOGICAL ADJUVANTS

(57) Abstract

The present invention features an adjuvanted vaccine, and methods for preparing an adjuvanted vaccine, preferably for immunizing against influenza, where the adjuvant is a lipid vesicle, and preferably is a nonphospholipid, paucilamellar lipid vesicle. The antigen may be encapsulated in the central cavity of the adjuvant, or mixed in solution with the adjuvant. Moreover, the adjuvant may carry a secondary adjuvant to further improve the immune response.

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Vaccines Containing Paucilamellar Lipid Vesicles As Immunological Adjuvants

Reference to Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/005,008 filed January 15, 1993, entitled *Method of Inhibiting Viral Reproduction*, the disclosure of which is incorporated herein by reference.

Background of the Invention

The present invention relates to an adjuvanted vaccine, where lipid vesicles, particularly nonphospholipid lipid vesicles, serve as the adjuvant, together with methods of preparing the vaccine. Immunological adjuvants are the component of the vaccine which augment the immune response to the antigen. Immunological adjuvants function by, inter alia, attracting macrophages to the antigen and then to present that antigen to the regional lymph nodes and initiate an effective antigenic response. Adjuvants may also act as carriers themselves for the antigen. Many of the known immunological adjuvants, such as Freund's complete adjuvant, alum, aluminum hydroxides, and Freund's incomplete adjuvant, while effective at initiating the antigenic response, produce undesirable reactions in humans, such as inflammation at the point of injection. These side effects prevent use of such adjuvants in humans, and have led to the search for alternative immunological adjuvants.

Lipid vesicles are substantially spherical structures made of amphiphiles, e.g., surfactants or phospholipids. The lipids of these spherical vesicles are generally organized in the form of lipid bilayers, e.g., multiple onion-like shells of lipid bilayers which encompass an aqueous volume between the bilayers. Certain types of lipid vesicles have an unstructured central cavity which can be used to encapsulate and transport a variety of materials. Paucilamellar lipid vesicles, for example, have 2-10 peripheral bilayers surrounding a large, unstructured central cavity.

Until recently, liposome technology has been concerned mostly with vesicles composed of phospholipids. This is primarily because phospholipids are the principal structural components of natural membranes and, accordingly, lipid vesicles have been used as a model system for studying natural membranes. However, there are a number of problems associated with using phospholipids as synthetic membranes. Phospholipid liposomes placed in an in vivo environment are rapidly degraded. Moreover, phospholipids are labile and expensive to purify or synthesize. In addition, classic phospholipid liposomes are in the form of multilamellar as opposed to paucilamellar vesicles and have poor carrying

capacities, especially for lipophilic materials, and have poor shelf lives unless lyophilized in the dark with antioxidants. Finally, phospholipids degrade too rapidly in vivo for most pharmaceutical or vaccine applications.

For these reasons, there is increasing interest in liposomes made of commercially available nonphospholipid amphiphiles (see, e.g., U.S. Pat. No. 4,217,344, U.S Pat. No. 4,917,951, and U.S. Pat. No. 4,911,928). These molecules have a hydrophilic head group attached to a hydrophobic "tail" and are derived from long chain fatty acids, long chain alcohols and their derivatives, long chain amines, and polyol sphingo- and glycerolipids. Commercially available amphiphile surfactants include, for example, the BRIJ family of polyoxyethylene fatty ethers, the SPAN sorbitan fatty acid esters, and the TWEEN polyoxyethylene derivatives of sorbitan fatty acid esters, all available from ICI Americas, Inc. of Wilmington, De. Paucilamellar vesicles containing such amphiphiles provide a high carrying capacity for water-soluble and water immiscible substances. The high capacity for water immiscible substances represents a unique advantage over classical phospholipid multilamellar liposomes.

Paucilamellar lipid vesicles may include a wide variety of phospholipids and nonphospholipid surfactants as their primary structural material. Paucilamellar lipid vesicles are substantially spherical structures made of materials having a high lipid content, preferably from nonphospholipid materials, which are organized in the form of lipid bilayers. The two to ten peripheral bilayers encapsulate an aqueous volume which is interspersed between the lipid bilayers and may also be encapsulated in the amorphous central cavity. Alternatively, the amorphous central cavity may be substantially filled with a water immiscible material, such as an oil or wax. Paucilamellar lipid vesicles have advantages as transport vehicles because large unstructured central cavity is easily adaptable for transport of large quantities of aqueous or oleaginous materials.

As described above, to stimulate a specific immune response, two components are required, namely the antigen or immunologicaly specific substance, and an adjuvant, the component augmenting the immune response to the antigen. Conventional adjuvants can serve as vehicles for the antigen, and as nonspecific immunological stimulants. The inventors have discovered that paucilamellar lipid vesicles are effective immunological adjuvants.

Accordingly, it is an object of the invention to provide an adjuvanted vaccine for immunizing against influenza, where paucilamellar lipid vesicles are the adjuvant.

Another object of the invention is to provide an adjuvanted vaccine to stimulate an immune response in a mammal, where the adjuvant is a nonphospholipid paucilamellar lipid vesicle which acts as a non-specific immune stimulator, an adjuvant/antigen carrier, or as a carrier of chemical adjuvants.

A further object of the invention is to provide a method of preparing adjuvanted vaccines useful in treating viral infections in mammals.

These and other objects and features of the invention will be apparent from the following description and from the claims.

Summary of the Invention

The present invention features an adjuvanted vaccine, and methods for preparing an adjuvanted vaccine, preferably for immunizing against influenza, where the adjuvant is a lipid vesicle, and preferably is a nonphospholipid, paucilamellar lipid vesicle. The antigen may be encapsulated in the central cavity of the adjuvant, or mixed in solution with the adjuvant. Moreover, the adjuvant may carry a secondary adjuvant to further improve the immune response.

The antigen is preferably an influenza antigen and may comprise a formalin-inactivated whole virus, formalin-inactivated viral subunits, or an antigen produced by recombinant DNA techniques.

In one embodiment, the adjuvanted flu vaccine is prepared whereby the paucilamellar lipid vesicles, the preferred adjuvant, are prepared separately, and the adjuvant is then intermixed with the antigen. Alternatively, an adjuvanted vaccine can be prepared by forming paucilamellar lipid vesicles encapsulating the antigen.

The adjuvant in one embodiment of the invention is a paucilamellar lipid vesicle having about two to ten bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. The lipid bilayers preferably have as their primary lipid component one or more of a the following nonphospholipid materials: polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof. More preferably, this mixture

further contains at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof, a charge producing agent, and any lipid soluble or water soluble materials to be incorporated into the vesicles.

The vesicles of the present invention have a central cavity, carrying either water soluble materials or a water-immiscible oily solution, which can be used to encapsulate the antigen. The water-immiscible oily solution is made of materials which are both water immiscible and immiscible in the lipids used to form the bilayers. The water immiscible oily material found the amorphous central cavity may comprise soybean oil, squalene oil, squalane oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof. These materials provide pharmacological benefits in addition to the benefits caused by the use of the particular lipids which form the bilayers.

The invention further features methods of producing adjuvanted vaccines. The adjuvant may comprise water or oil filled vesicles, e.g., vesicles having their amorphous central cavities filled with a water-immiscible oily solution, and these may be formed using either the "hot loading" technique disclosed in Unites States Patent No. 4,911,928 or the "cold loading" technique described in the United States Patent No. 5,160,669, the disclosures of which are incorporated herein by reference. In either case, a lipid phase is formed by blending the nonphospholipid material, along with any sterols or lipophilic materials to be incorporated into the lipid bilayers, to form a homogenous lipid phase. In the "hot loading" technique, any water-immiscible oily material to be encapsulated in the vesicles is blended in the already formed lipid phase, forming a lipophilic phase. Oil-soluble or oil-suspendable antigens to be encapsulated within the vesicles are first dispersed in the oil. The term "dispersed" as used herein includes dissolution or forming a suspension or colloid to yield a flowable phase.

Once a lipophilic phase is made, it is blended with an aqueous phase (e.g., water, saline, or any other aqueous solution which will be used to hydrate the lipids), which may also contain an antigen, under shear mixing conditions to form the adjuvant. "Shear mixing conditions", as used herein, means a shear equivalent to a relative flow of 5-50 m/s through a 1mm orifice.

In the alternative, the vaccine can be incorporated into the amorphous central cavity of the adjuvant by the "cold-loading" technique described in U.S. Patent No. 5,160,669 to Wallach et al.

The scope and application of the invention will be apparent from the following detailed description.

Brief Description of the Drawings

FIG. 1 is a graph of the mean IFA results in mice at day 42 following one inoculation with adjuvanted influenza A vaccines;

FIG. 2 illustrates the mean IFA values in rabbits at day 27 following two inoculations with adjuvanted influenza A vaccines;

FIG. 3 illustrates the mean HI values in rabbits at day 27 following two inoculations with adjuvanted influenza A vaccines.

Detailed Description of the Invention

The present invention involves use of paucilamellar lipid vesicles as adjuvants in a vaccine to increase the antigenic response in a mammal inoculated with the vaccine. The vesicles are preferably nonphospholipid vesicles, and the antigen is preferably an influenza antigen.

Paucilamellar lipid vesicles act to stimulate the immune response several ways, as non-specific stimulators, as carriers for the antigen, as carriers of additional adjuvants, and combinations thereof. Paucilamellar lipid vesicles act as non-specific immune stimulators when, for example, a vaccine is prepared by intermixing the antigen with the preformed vesicles such that the antigen remains extracellular to the vesicles. By encapsulating an antigen within the central cavity of the vesicle, the vesicle acts both as an immune stimulator and a carrier for the antigen. Alternatively, the vesicles can act as carriers for the antigen by fusing with the antigen, as is described in U.S. Patent Application Serial No. 08/005,008 filed January 15, 1993, entitled *Method of Inhibiting Viral Reproduction*, of which this application is a continuation-in-part. In this embodiment, when the antigen, there an enveloped virus, is mixed with the paucilamellar lipid vesicles, the virus and adjuvant fuse, to denaturing the nucleic acid and inactivating the virus. The inactivated virus/adjuvant hybrid is then useful as a vaccine. Moreover, the vesicle can serve to carry additional adjuvants within the central cavity or between the bilayers.

The following Examples will clearly illustrate the efficacy of the invention.

Example 1:

An adjuvanted vaccine containing the antigen influenza A H3N2 (Beijing) was prepared using nonphospholipid paucilamellar lipid vesicles as adjuvants. Adjuvanticity of the two formulations, namely, non-specific immune stimulator and carrier adjuvant formulations was compared using the mean Indirect Fluorescent Assay (IFA) of each composition, as compared with that of the antigen alone, as shown in Figure 1.

Adjuvant formulations were prepared using an automated syringe machine, specifically a 5cc syringe machine. The adjuvant could also be made according to the general procedure set forth in United States Patent No. 4,911,928. Briefly, the lipid components of the vesicle walls were heated to a flowable state and placed in a first component of the syringe machine. The aqueous component, in this case containing the antigen Fluzone™ (see below), was heated and placed in a second component of the syringe machine. The materials were then mixed using shear mixing until vesicles formed, encapsulating the antigen in the central cavity. However, in this and the following Examples, any method of achieving the proper shear could be used, including the manual techniques described in U.S. Patent No. 4,911,928 (two syringes connected via a stopcock), or a flow device such as the NovaMix™ vesicle former. The basic details of the NovaMix™ system are described in United States Patent No. 4,895,452, the disclosure of which is incorporated herein by reference.

The antigen used in this example was Fluzone™ a formalin-inactivated detergent-extracted influenza vaccine from Connaught. The formulation for the adjuvants used in this Example are set forth in Tables 1 and 2 below.

TABLE 1

Lipid Formulation	Brij 52 (7.0g); cholesterol (2.69g)
Diluent	Water for injection (WFI) containing 2.4 μg/ml Fluzone
Diluent Volume	4.0ml
Charge	Negative (Oleic acid 0.31g)
Oil	Squalene
Hydration Ratio	1.6/1 (lipid/sqe) 1.0/4.0 (lipid, oil/Diluent)
Temperature of WFI Phase	60° C
Temperature of Lipid Phase	85° C
pH	5.85
Final Volume	5 ml

For the first vaccine preparation, where the adjuvant encapsulates the antigen, the vaccine was made according to the formula of Table 1. The second vaccine preparation is made according to the formula of Table 2 below, where the diluent is water, without the antigen.

TABLE 2

Lipid Formulation	Brij 52 (7.0g); cholesterol (2.69g)
WFI	Water for injection (WFI)
Diluent Volume	4.0ml
Charge	Negative (Oleic acid 0.31g)
Oil	Squalene
Hydration Ratio	1.6/1 (lipid/sqe); 1.0/4.0 (lipid, oil/Diluent)
Temperature of WFI Phase	60° C
Temperature of Lipid Phase	85° C
pH	5.85
Final Volume	5 ml

The adjuvant for the second vaccine preparation is prepared according to the method described above and then diluted 1:10. Of that diluted adjuvant, 100 μl are added to 2.4 μg of the Fluzone antigen for injection into each animal.

Three groups of ten C_3H seven week old female mice where injected with each vaccine preparation, resulting in 2.4 micrograms of antigen given per mouse. The first group of mice received one injection of the antigen alone; the second group received one injection of the antigen incorporated into the adjuvant; and the third group of mice received one injection of the antigen intermixed with the one to ten dilution of adjuvant. As can be seen from FIG. 1, which illustrates mean IFA results at day 42, the adjuvanted vaccines improved the antigenic response significantly over the antigen alone. The adjuvant encapsulating the antigen exhibits a log increase over the antigen alone, and the diluted adjuvant exhibits a 7/10 log increase.

Example 2:

In this example, New Zealand Albino rabbits from Hazelton Labs were immunized with adjuvanted influenza A (Beijing) H3N2 vaccines to compare the adjuvant formulations of the present invention with the antigen alone, and with two other adjuvants not suitable for use in humans.

Each group of six rabbits (three males and three females) was injected with 9.8 micrograms of influenza A H3N2 antigen per animal. This antigen is a whole virus preparation produced in chicken eggs, which has been formalin-inactivated and purified by centrifugation and column filtration. In each case, one half milliliter of the vaccine was injected intramuscularly into each rabbit at days 0 and 14. The data from Figures 2 and 3 was determined from a bleeding taken on day 27.

The first group of rabbits received the antigen alone, the second group of rabbits received the antigen adjuvanted with 16 μg alum/1μg protein (Resorptar Armour Pharmaceuticals). The third group received the antigen adjuvanted with a 1:1 mixture (vol/vol) incomplete Freund's (Sigma Chemical).

The fourth group of rabbits received the antigen encapsulated in paucilamellar lipid vesicles prepared according to the formula set out in Table 3 below, prepared as described in Example 1, and the fifth group received 9.8 μg antigen in solution 1:1 (vol/vol) with adjuvant, specifically the paucilamellar lipid vesicles prepared according to the formulation set forth in Table 4 below.

TABLE 3

Lipid Formulation	Brij 52 (17.5g), cholesterol (6.4g)
Diluent	Water for injection (WFI) with 9.8 μg antigen
Diluent Volume	3.7ml
Charge	None
Oil	Soybean oil
Hydration Ratio	(1.4ml lipid/1ml oil); 1.3/3.7 (lipid/oil to WFI)
Temperature of WFI Phase	56°C
Temperature of Lipid Phase	74° C
pH	6.6
Final Volume	5 ml

TABLE 4

Lipid Formulation	Brij 52 (17.5g), cholesterol (6.4g)
Diluent	Water for injection (WFI)
Diluent Volume	3.7ml
Charge	None
Oil	Soybean oil
Hydration Ratio	(1.4ml lipid/1ml oil); 1.3/3.7 (lipid/oil to WFI)
Temperature of WFI Phase	56° C
Temperature of Lipid Phase	74° C
pH	6.6
Final Volume	5 ml

As can be seen from Figures 2 and 3, the adjuvanted vaccine according the present invention has equivalent or increased antibody response and antigenicity when compared with that of known adjuvants. The mean IFA results were calculated as described above. The mean HI values, Hemagglutination Inhibition assay, were obtained from testing with chicken red blood cells, as is known in the art, the results of which correlate with protection capabilities of the vaccine.

The foregoing Examples are merely illustrative and those skilled in the art may be able to determine other materials and methods which accomplish the same result. Such other materials and methods are included within the scope of the following claims.What is claimed is:

1. A vaccine for producing an antigenic response to influenza, in vivo, in mammals, said vaccine comprising:

an effective amount of an influenza antigen and an adjuvant, said adjuvant comprising paucilamellar lipid vesicles having nonphospholipid materials as the primary wall forming constituent.

2. The vaccine of claim 1 wherein said antigen and said adjuvant are intermixed in said vaccine.

3. The vaccine of claim 1 wherein said antigen is encapsulated in said adjuvant.

4. The vaccine of claim 1 wherein said paucilamellar lipid vesicles have 2-10 bilayers surrounding an amorphous central cavity.

5. The vaccine of claim 4 wherein said antigen is encapsulated in said amorphous central cavity.

6. The vaccine of claim 1 wherein said antigen is selected from the group consisting of antigens derived from formalin-inactivated whole virus, antigens derived from formalin-inactivated viral subunits, and antigens produced by recombinant DNA techniques.

7. The vaccine of claim 6 wherein said antigen is selected from the group consisting of Fluzone and influenza A H3N2.

8. The vaccine of claim 1 wherein said nonphospholipid materials are selected from the group consisting of polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof.

9. The vaccine of claim 1 wherein said paucilamellar lipid vesicles further comprise at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

10. The vaccine of claim 1 wherein said paucilamellar lipid vesicles comprise an amorphous central cavity containing a water immiscible oily material.

11. The vaccine of claim 10 wherein said water immiscible oily material is selected from the group consisting of soybean oil, squalene oil, squalane oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof.

12. A method of immunizing a mammal against influenza, said method comprising the step of administering to said mammal a vaccine comprising an influenza antigen and an adjuvant, wherein said adjuvant comprises nonphospholipid paucilamellar lipid vesicles.

13. The method of claim 12 wherein said administering step is selected from the group consisting of administering said vaccine intramuscularly, intraperitonealy, and orally.

14. The method of claim 12 wherein said antigen and said adjuvant are intermixed in said vaccine.

15. The method of claim 12 wherein said antigen is encapsulated in said adjuvant.

16. The method of claim 12 wherein said paucilamellar lipid vesicles have 2-10 bilayers surrounding an amorphous central cavity.

17. The method of claim 16 wherein said antigen is encapsulated in said amorphous central cavity.

18. The method of claim 12 wherein said antigen is selected from the group consisting of antigens derived from formalin-inactivated whole virus, antigens derived from formalin-inactivated viral subunits, and antigens produced by recombinant DNA techniques.

19. The method of claim 12 wherein said antigen is selected from the group consisting of Fluzone and influenza A H3N2.

20. The method of claim 12 wherein said nonphospholipid paucilamellar lipid vesicles comprise nonphospholipid materials selected from the group consisting of polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof.

21. The method of claim 12 wherein said paucilamellar lipid vesicles further comprise at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

22. The method of claim 12 wherein said paucilamellar lipid vesicles comprise an amorphous central cavity containing a water immiscible oily material.

23. The method of claim 22 wherein said water immiscible oily material is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof.

24. A method of preparing an adjuvanted influenza vaccine, said method comprising the steps of:

forming an adjuvant comprising paucilamellar lipid vesicles having 2-10 lipid bilayers surrounding an amorphous central cavity; and

mixing said adjuvant with an influenza antigen.

25. The method of claim 24 wherein said antigen is encapsulated in said amorphous central cavity of said adjuvant.

26. The method of claim 24 wherein said antigen is selected from the group consisting of antigens derived from formalin-inactivated whole virus, antigens derived from formalin-inactivated viral subunits, and antigens produced by recombinant DNA techniques.

27. The method of claim 24 wherein said antigen is selected from the group consisting of Fluzone and influenza A H3N2.

28. The method of claim 24 wherein said paucilamellar lipid vesicles comprise nonphospholipid materials selected from the group consisting of polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof.

29. The method of claim 24 wherein said paucilamellar lipid vesicles further comprise at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

30. The method of claim 24 wherein said paucilamellar lipid vesicles comprise an amorphous central cavity containing a water immiscible oily material.

31. The vaccine of claim 33 wherein said water immiscible oily material is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof.

32. A method of preparing an adjuvanted influenza vaccine, said method comprising the steps of:

preparing a lipophilic phase containing a nonphospholipid material;

preparing an aqueous phase containing an influenza antigen; and

shear mixing said lipophilic phase with said aqueous phase to form paucilamellar lipid vesicles having 2-10 lipid bilayers surrounding an amorphous central cavity, wherein said antigen is encapsulated in said cavity.

33. The method of claim 32, wherein said antigen is selected from the group consisting of antigens derived from formalin-inactivated whole virus, antigens derived from formalin-inactivated viral subunits, and antigens produced by recombinant DNA techniques.

34. The method of claim 32 wherein said antigen is selected from the group consisting of Fluzone and influenza A H3N2.

35. The method of claim 32 wherein said nonphospholipid material is selected from the group consisting of polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof.

36. The method of claim 32 wherein said lipophilic phase further comprises at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

37. The method of claim 32 wherein said amorphous central cavity of said paucilamellar lipid vesicles contains a water immiscible oily material.

38. The method of claim 37 wherein said water immiscible oily material is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof.

39. A method of forming an adjuvanted vaccine wherein said adjuvant comprises a paucilamellar lipid vesicle having a substantially amorphous central cavity containing a water-immiscible oily material and an antigen, said method comprising the steps of:

preforming a paucilamellar lipid vesicle having an aqueous material in the amorphous central cavity; and

mixing under shear mixing conditions, said preformed paucilamellar lipid vesicle with a water immiscible material containing an antigen to be incorporated into said central cavity such that said water immiscible material and said antigen are incorporated into said preformed vesicle, whereby said amorphous central cavity of said paucilamellar lipid vesicle is substantially filled with said water immiscible material.

40. The method of claim 39 further comprising the step of separating said paucilamellar lipid vesicle from any of said water immiscible material and antigen not incorporated into said paucilamellar lipid vesicle.

41. The method of claim 39 wherein said paucilamellar lipid vesicles have 2-10 bilayers surrounding an amorphous central cavity.

42. The method of claim 39, wherein said antigen is selected from the group consisting of antigens derived from formalin-inactivated whole virus, antigens derived from formalin-inactivated viral subunits, and antigens produced by recombinant DNA techniques.

43. The method of claim 39 wherein said antigen is selected from the group consisting of Fluzone and influenza A H3N2.

44. The method of claim 39 wherein said paucilamellar lipid vesicle comprises nonphospholipid materials selected from the group consisting of polyoxyethylene fatty acid esters, polyoxyethylene fatty acid ethers, polyoxyethylene sorbitan esters, polyoxyethylene glyceryl mono- and diesters, glyceryl mono-and distearate, sucrose distearate, propylene glycol stearate, long chain acyl hexosamides, long chain acyl amino acid amides, long chain acyl amides, glyceryl mono-and diesters, dimethyl acyl amines, C_{12}-C_{20} fatty alcohols, C_{12}-C_{20} glycol monoesters, C_{12}-C_{20} fatty acids, and mixtures thereof.

45. The method of claim 39 wherein said paucilamellar lipid vesicle further comprise at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

46. The method of claim 39 wherein said water immiscible oily material is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof.

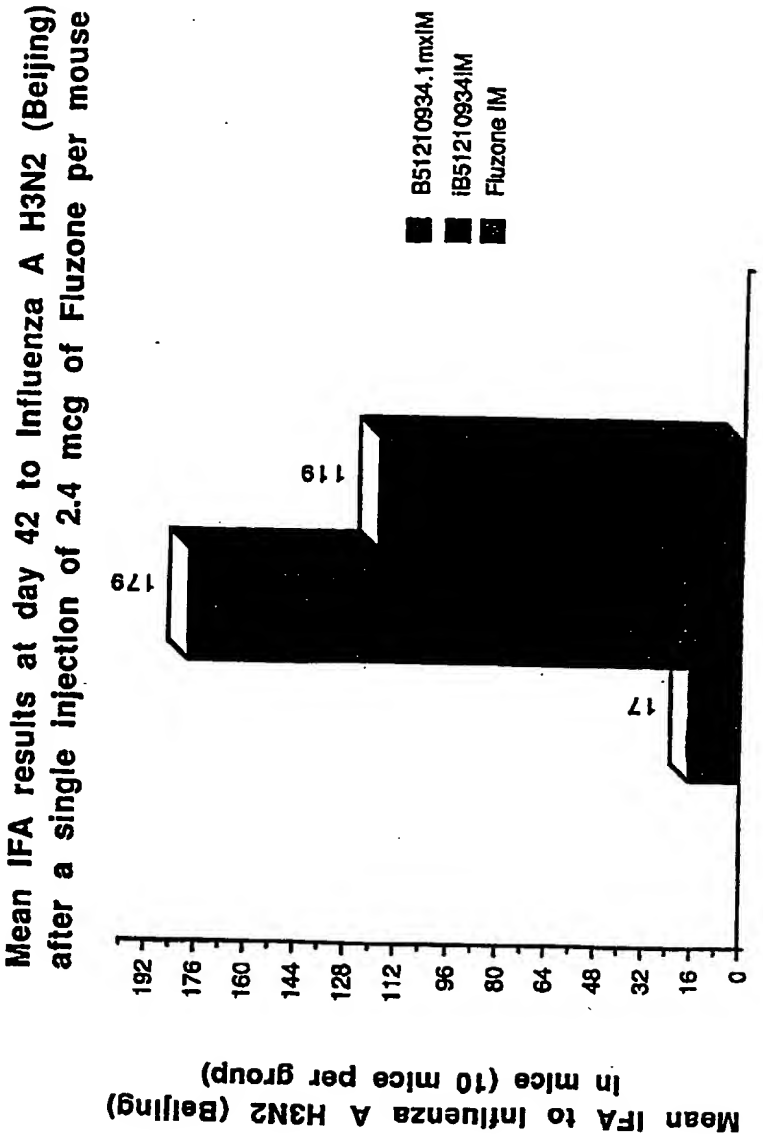
Mean IFA results at day 42 to Influenza A H3N2 (Beijing) after a single injection of 2.4 mcg of Fluzone per mouse
B51210934.1mxIM
iB51210934IM
Fluzone IM
179
119
17
192
176
160
144
128
112
96
80
64
48
32
16
0
Mean IFA to Influenza A H3N2 (Beijing) In mice (10 mice per group)

FIG. 1

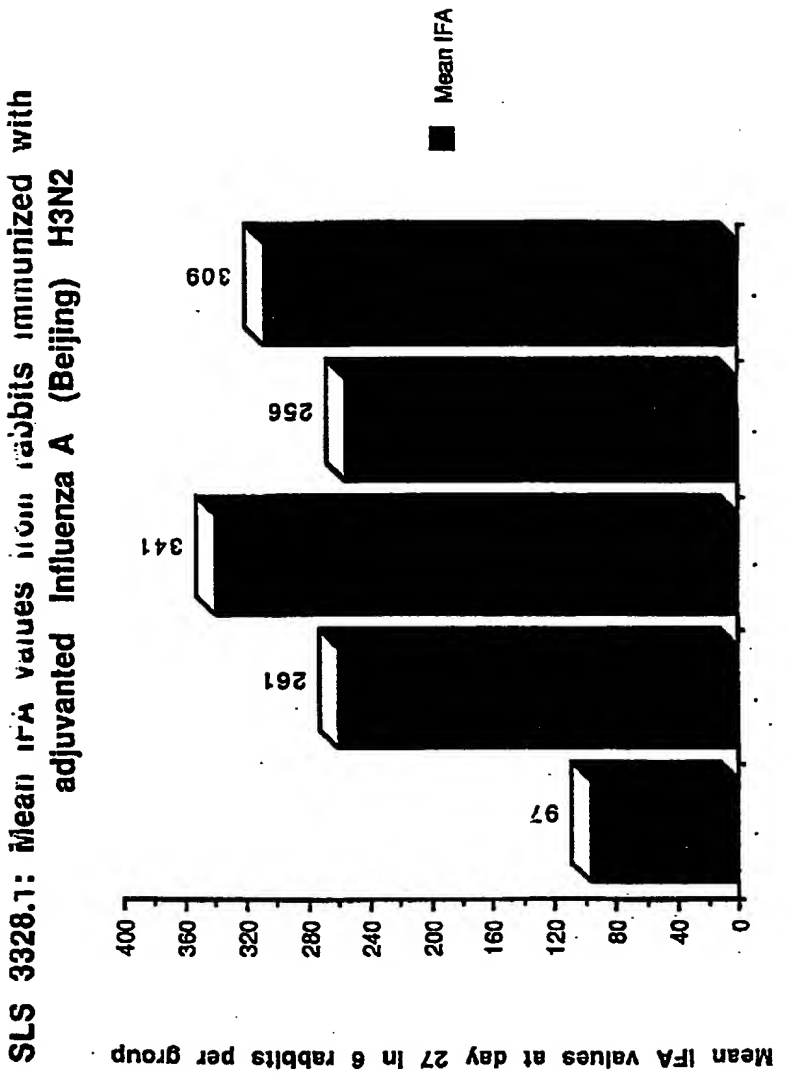
SLS 3328.1: Mean IFA values from rabbits immunized with adjuvanted Influenza A (Beijing) H3N2
Mean IFA
Mean IFA values at day 27 in 6 rabbits per group
400
360
320
280
240
200
160
120
80
40
0
97
261
341
256
309
Flu Alone
Alum-Flu
IFA-Flu
Flu in Nova
Flu mx Nova

FIG. 2

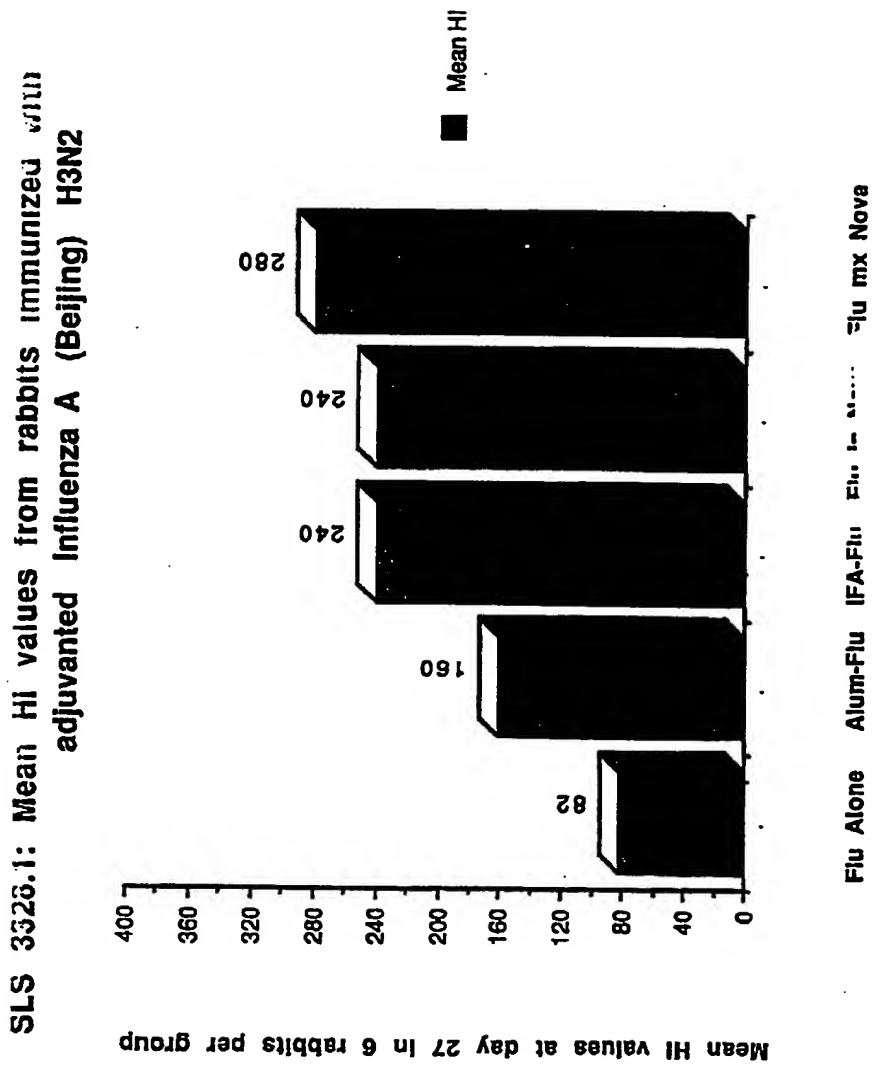
SLS 3326.1: Mean HI values from rabbits immunized with adjuvanted Influenza A (Beijing) H3N2
Mean HI
Mean HI values at day 27 in 6 rabbits per group
400
360
320
280
240
200
160
120
80
40
0
82
160
240
240
280
Flu Alone
Alum-Flu
IFA-Flu
Flu mx Nova

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00475

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/450.1, 209.1, 210.1, 211.1, 184.1, 420; 428/402.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450.1, 209.1, 210.1, 211.1, 184.1, 420; 428/402.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,911,928 (WALLACH) 27 MARCH 1990, abstract, column 5, lines 33-47, column 3, lines 22-45, column 3, lines 58 to column 4, lines 33, column 6, lines 23-28, column 3, lines 48-60, column 4, lines 47-63.	1-11, 24-46
Y	US, A, 5,000,960 (WALLACH) 19 March 1991, abstract, column 1, lines 61-68, column 5, lines 54-65, column 4, lines 63 to column 5, lines 25, column 6, lines 30-44, examples, claims.	1-46
Y	EP, A, 0,356,340 (POPESCU ET AL.) 28 February 1990, abstract, page 3, lines 57-60, page 3, lines 61-65, page 4, lines 34-44, page 5, lines 52-57.	1-46

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 MARCH 1995	Date of mailing of the international search report 31 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NITA M. MINNIFIELD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00475

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,100,662 (BOLCSAK ET AL.) 31 March 1992, abstract, column 9, line 53 to column 10, line 20.	1-46
Y	US, A, 4,241,046 (PAPAHADJOPOULOS) 23 December 1980, see entire document.	1-46
A	US, A, 5,234,767 (WALLACH) 10 August 1993, see entire document.	1-46
A	US, A, 5,147,723 (WALLACH) 15 September 1992, see entire document.	1-46
A	US, A, 5,032,457 (WALLACH) 16 July 1991, see entire document.	1-46
Y	US, A, 4,826,687 (NEROME ET AL.) 02 May 1989, see entire document.	1-46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00475

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/145, 39/155, 39/00, 39/38; B28B 1/00; B29C 35/02; B32B 9/00, 9/02, 9/04

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

HCA, WPIDS, MEDLINE, BIOSIS, LIFESCI, CONFSCI, EMBASE, IFIPAT, DISSABS, APS
search terms: paucilamellar, influenza, lipid vesicles, vaccine, inventor names, adjuvant, polyoxyethylene esters, glyceryl monostearate, glyceryl distearate, sucrose